Identification and Detection of *omp*W Gene in *Vibrio cholerae* Isolates from Raw Meat and Street Vended Food in Kuching, Sarawak

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LIST OF ABBREVIATIONS

A	Acid
CT	Cholera Toxin
H_2S	Hydrogen sulphite
Hr(s)	Hour(s)
K	Alkaline
Min(s)	Minute(s)
ml	Mililiter
NaCl	Sodium chloride
s	Second(s)
sp./spp.	Species
v/v	Volume/Volume
w/v	Weight/Volume
TSI	Triple Sugar Iron
PCR	Polymerase Chain Reaction
MR	Methyl Red
NA	Nutrient Agar
NaCl	Sodium chloride
NB	Nutrient Broth
V. cholerae	*Vibrio cholerae*
VP	Voges-Proskauer
OMP	Outer membrane protein
*omp*W	Outer membrane protein W
LPS	Liposaccharides
WHO	World Health Organization
%	Percent
ºC	Degree Celsius

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ABSTRACT

Vibrio cholerae is a pathogenic bacterium which causes cholera if ingested through consumption of contaminated food such as raw meat and street vended food. Outer membrane proteins of *V.cholerae* play an important role during infection and induction of host commonly to human. A study was carried out for the identification of *V. cholerae* and detection of *omp*W gene in *V. cholerae*. Gram-staining and series of biochemical tests were performed for the identification of the *V. cholerae* isolates. Biochemical test showed ambiguity results. In this study, PCR assay for the detection of *omp*W gene in *V. cholerae* involved 5 samples of raw meat and 19 samples of street vended food. *Omp*W gene was only detected in the positive control of *V. cholerae* while there was no detection of *omp*W in all of the samples. This study shows a low occurrence of *V. cholerae* meat and street vended food in Kuching, Sarawak. Further research on this pathogenic bacterium is needed to avoid infection due to the consumptive of contaminated food.

Key words: *Vibrio cholerae*, biochemical tests, Gram-staining, PCR, *omp*W gene

ABSTRAK

Vibrio cholerae merupakan bakteria patogen yang menyebabkan penyakit cirit-birit jika memakan daging mentah dan makanan yang dijual di tepi jalan yang tercemar. Protein pada lapisan kulit luar V. cholerae memainkan peranan yang penting semasa infeksi dan induksi terhadap perumah terutamanya manusia. Kajian telah dilakukan untuk pengecaman gen ompW pada V. cholerae. Teknik pewarnaan Gram dan beberapa siri ujian biokimia telah dilakukan. Kaedah PCR digunakan untuk mengesan gen ompW di dalam V. cholerae melibatkan 5 sampel daripada daging mentah dan 19 sampel daripada makanan yang dijual di tepi jalan. Keputusan menunjukkan gen ompW hadir dalam kawalan positif tetapi tiada dalam semua sampel yang lain. Kajian ini menunjukkan bahawa, kadar kehadiran V. cholerae dalam daging mentah dan makanan yang dijual di tepi jalan adalah rendah. Kajian selanjutnya berkenaan bakteria pathogen ini adalah perlu untuk mengelakkan jangkitan melalui pemakanan makanan yang tercemar.

Kata kunci: *Vibrio cholerae, ujian biokimia, pewarnaan Gram, PCR, gen ompW*

CHAPTER 1

INTRODUCTION

1.1 Introduction

Vibrio cholerae are typically responsible for the diarrheal disease known as cholera. *V. cholerae* belongs to a group of organisms whose natural habitats are the aquatic ecosystems. Cholera usually spread by poor sanitation, resulting in contaminated water supplies. Therefore, cholera disease is easily contracted by humans if humans consume the contaminated water. This is clearly the main means for the spread of cholera in poor communities such as in South America (Mintz *et al*., 1994). According to World Health Organization (WHO), the number of cases reported in 1993, was 376 845 in 78 countries with 6781 deaths (Said and Drasar, 1996).A recent study of Khuntia (2008) stated that, cholera has been reported in the state of Orissa, India during the past decades. An outbreak of diarrheal disease occurred during November 1 to November 9, 2000 in Rusipada village near Puri.

Transmission of *V. cholerae* to humans occurs through ingesting contaminated water or food. Cholera epidemics caused by toxigenic *V. cholerae* represent a major public health problem in developing countries like India and Bangladesh (Thomas *et al*., 2008). Poultry meat and poultry products such as eggs are the most popular dishes for the people in Bangladesh. This is because; processed poultry meat and eggs are their main sources of protein. However, poultry and poultry products are considered as the major infectious routes for humans because different species of pathogenic and non-pathogenic microorganisms have been reported in poultry (Akond *et al*., 2008).

A study by Ronghua (2008) stated that, outer membrane proteins of Gram-negative bacteria play an important role during infection and pathogenicity to host. The outer membrane protein of Gram-negative bacteria is a protective barrier that hinders the permeability of hydrophobic and hydrophilic compounds because of presence of liposaccharides (LPS) within the outer leaflet of the outer membrane. A recent study of Heedeok (2006) says that, the function of *omp*W is unknown but they may be involved in the protection of bacteria against the various form of environment stress.

The increasing spread of cholera due to food consumption has been a concern to the nations of the world. Thus this study is conducted to identify and to detect the *omp*W gene in *V. cholerae* from food sources such as meat and street vended food. The main experiments in this study are purification of *V. cholerae* isolates, biochemical tests and Gram-staining, DNA extraction used as templates for PCR amplification and PCR amplification for detection of *omp*W in *V. cholerae.*

1.2 Objectives

The objectives of this study are:

1. To identify the isolated strains of *Vibrio cholerae* by biochemical test.
2. To determine the morphology of the bacterial on selective media and by Gram-staining.
3. To detect the *omp*W gene in *Vibrio cholerae* by PCR assay.

CHAPTER 2

LITERATURE REVIEW

2.1 *Vibrio cholerae*

There are two varieties of *V. cholerae* that are potentially pathogenic to humans. The main type causing cholera is *V. cholerae* O1 serotype, the other types *V. cholerae* is non-O1 serotype. *V. cholerae* O1 is responsible for epidemic and pandemic cholera. Isolates of *V. cholerae* can be divided into two biotypes, El Tor and classical, on the basis of several phenotypic characteristic. Within the O1 serogroup, the ability to produce cholera toxin (CT) is an essential determinant of virulence. *V. cholerae* non-O1 can possess a variety of possible virulence factors; including production of CT. This bacterium of non-O1 serotype infects only humans and other primates (Kay *et al*., 1994).

2.1.1 Characteristic of *Vibrio cholerae*

V. cholerae is the type species of the genus Vibrio, which is the type genus of the family *Vibrionaceae*. *Vibrio* spp. requires salt such as NaCl for their growth. *Vibrio cholerae* also requires 5 to 15 mM Na^+ for optimum growth but usually grow in complex medium for example in nutrient broth which does not require the addition of salt. *V. cholerae* will grow in alkaline conditions up to pH10 but is inhibited when the pH drops to 6.0 and below (Kay *et al*., 1994). *Vibrio cholerae* gives positive reactions in lysine, ornithine, citrate utilization, nitrate reduction, lipase, gelatinase, oxidase fermentation tests but are negative in arginine, urease and luminescence tests. They are able to grow in nutrient broth containing 0, 3 and

6 % of sodium chloride but will not grow in nutrient broth containing 8% and above of NaCl. These strains produce acid from glucose, lactose and sucrose. All strains do not produce gas from glucose (Hoa, n.d.). *Vibrio cholerae* is a Gram-negative, highly motile with a single polar flagellum, curved or comma-shaped rod bacterium that produces cholera enterotoxin and responsible for the life-threatening secretory diarrhea (Madden *et al*., 1989).

2.1.2 Taxonomy

The Family *Vibrionaceae* is found in the "Facultative Anaerobic Gram-negative Rods" in Bergey's Manual (1986), on the level with the Family *Enterobacteriaceae*. In the revisionist taxonomy of 2001 (Bergey's Manual), based on phylogenetic analysis, *Vibrionaceae, Pseudomonadaceae and Enterobacteriaceae*are all landed in the *Gammaproteobacteria.*

2.2 Cholera

Cholera is an acute intestinal infection caused by *Vibrio cholerae* characterized by a severe diarrheal disease caused by the bacterium. Transmission to humans is by water or food. *Vibrio cholerae* is transmitted through water contaminated with fecal matter (Goel *et al*., 2007). Foodborne infections have been traced to raw or inadequately cooked shellfish and other seafood. In its extreme manifestation, cholera is one of the most rapidly fatal illnesses known. A healthy person may become hypotensive within an hour of the onset of symptoms and may die within 2-3 hours if no treatment is provided (Koch *et al*., 1993).

2.3 Outer membrane protein W (*omp*W) genes

*Omp*W belongs to a family of small OMPs which are widespread in Gram-negative bacteria. It is a major antigen in bacterial infections and host immune response. *Omp*W forms eight-stranded β-barrel with a long and narrow hydrophobic channel (Heedeok *et al*., 2006). A study has been performed for the distribution of *omp*W genes in *Vibrio cholerae* using respective primers and probes. PCR amplification results showed that all of the *V. cholerae* strains tested were positive for *omp*W gene (Nandi *et al*., 2000).

2.4 Biochemical Tests

2.4.1 IMViC

IMViC is an acronym that stands for indole, Methyl Red and Voges-Proskauer and citrate test. The indole test utilizes SIM media to identify the capabality of bacteria producing indole using the enzyme tryptophanase. When Kovac's reagent is added into the inoculated media, a dark pink color develops for positive result. The indole test must be read by 48 hours of incubation because the indole can be further degraded if prolonged incubation occurs. MRVP is performed after 48 hours of incubation with inoculated bacterial. Methyl Red (MR) test identify bacteria that produce stable acid end products by mean of mixed acid fermentation of glucose. The reagent used for VP test are Barritt's A (alpha napthol) and Barritt's B (potassium hydroxide). When these reagents are added into a broth, they turn into a pink-burgandy color for a positive result. This color might take 20-30 minutes to develop. The citrate test utilizes Simmon's citrate slant to determine if bacteria can grow utilizing citrate as its sole carbon and energy source. Growth of media in the media leads to development of Prussian blue color and this shows a positive result (IMViC, 1998).

2.4.2 Triple Sugar Iron (TSI) Agar

Triple Sugar Iron media contains three sugars; glucose, lactose and sucrose; the pH indicator phenol red detecting carbohydrate fermentation indicated by the production of gas and a change in the color of the pH indicator from red to yellow, and ferrous ammonium sulfate for detection of hydrogen sulfide production indicated by blackening in the butt of the tube (Chamberlain, 2000).

2.4.3 Salt Tolerance Test

The salt tolerance test is to identify the growth of *V. cholerae* in different percentage of NaCl. *V. cholerae* will grow in nutrient broth which contains 0-3 % NaCl but some strains may grow in broth contains 6 % NaCl. However, they do not grow in nutrient broth contains 8 % NaCl and above (Choopun *et al*., 2000).

2.4.4 Oxidase Test

The oxidase test also known as Cytochrome Oxidase Test identifies organisms that produce the enzyme cytochrome oxidase. The oxidase test reagent contains a chromogenic reducing agent, which is a compound that changes color when it becomes oxidized. If the test organism produces cytochrome oxidase, the oxidase reagent will turn blue or purple within 15 seconds (Oxidase test, 2005).

2.4.5 Gram-staining

This method was developed by Hans Christian Gram in 1884. The purpose of Gram-staining is to differentiate between Gram-positive and Gram-negative bacteria species. Bacteria have distinct and consistent differences in their cell walls based on their chemical and physical properties of their cell walls. Gram-positive bacteria would appear violet while Gram-negative bacteria would appear pink color (Rollins and Joseph, 2000).

2.5 Polymerase Chain Reaction

Developed by Kary Mullis in 1983, Polymerase Chain Reaction (PCR) is a molecular technique for the amplification of a single or a few copies of DNA molecule producing thousands to millions of copies of particular DNA sequence. This method is consisting of cycles of heating and cooling of the PCR mixture. These thermal cycling involves first, thermal denaturation of the DNA double helix at high temperature, follow by the annealing of the primers to the separated strands at lower temperature and finally, elongation. A pair of oligonucleotide primers flanking the target sequence to be amplified. The *Taq* DNA polymerase catalyzes the DNA synthesis. The wide spread success of PCR as a technique comes from its speed, efficiency, and reproducible of the reaction making it suitable to many procedures in basis science and pathology laboratory (Brand, 1995).

According to Koch (1993), foods such as, oysters, crabmeat, shrimp, and lettuce were seeded with *V. cholerae* and a rapid PCR method has been performed for the detection of *Vibrio cholerae* in foods. *Vibrio cholerae* has cause diarrheal outbreaks in Hong Kong. Therefore, a study has been developed by using PCR method to aim for the detection of food-borne pathogens in clinical specimens, food and environmental samples (Ling, 2009).

CHAPTER 3

MATERIAL AND METHODS

3.1 Sources of *Vibrio cholerae* and bacterial isolates

The *V. cholerae* stains are available in the Microbiology laboratory, UNIMAS which have been isolated from the raw meat and street vended food. The isolates of *V. cholerae* which are stored in LB broth supplement with 15% glycerol (v/v) were grown in Luria Bertani (LB) broth overnight at 37°C with shaking at 150 rpm in an orbital shaker (Lab-line Incubator-shaker).

3.2 Purification of *Vibrio cholerae* isolates

A loop of culture from the LB broth was streaked on the TCBS agar surface. The plates were incubated overnight in 37°C incubator. Pure yellow colonies of *V. cholerae* isolates were selected and were streaked onto the nutrient agar slant as working cultures and stock culture. These working cultures were used for the subsequent analysis.

3.3 Biochemical Tests and Gram-staining

These biochemical tests were performed according to Bergey's Manual (1984). Six biochemical tests were performed for each isolate. A loop of isolate from the nutrient slant working culture were streaked on the nutrient agar plate and incubated at 37ºC overnight.

3.3.1 MRVP Test

A single bacterial colony was inoculated into a single tube of MR-VP broth with sterile loop. The culture was incubated at 37ºC for 48 hours. After the culture was grown, about half of the culture was transferred into a clean tube. Methyl red was added into the first tube for MR test. A red color indicates a positive result while a yellow color indicates a negative result. First a Barritt'sA (alpha-napthol) and then Barritt's B (potassium hydroxide) reagents were added into the second tube for VP test. Changes of color were observed after about 15 minutes. A red color indicates a positive result while a yellowish to copper color indicates a negative result.

3.3.2 The Citrate Test

Simmon's citrate media were prepared in screw cap tubes in a slanted position. A single bacterial colony was inoculated with sterile loop and streaked along the surface of the slant. The inoculated tubes were incubated with screw caps loosened at 37ºC for 18-24 hours. This test was performed to determine the ability of the bacterial to utilize citrate as its sole carbon source. A blue color was considered a positive result while no color change was considered a negative result.

3.3.3 Carbohydrate Fermentation Test

The TSI media was prepared in a slanted position. A single bacterial colony was inoculated with sterile needle and stabbed in the butt of the tube and then streaked back and forth along the surface of the slant. The inoculated tubes were incubated with caps loosened at

37ºC and examined after 18-24 hours for carbohydrate fermentation, gas production and hydrogen sulfide production.

3.3.4 Indole Test

The bacterial colonies were inoculated with sterile needle and stabbed into the SIM media. The bacteria were incubated for 48 hours. After incubation, Kovac's reagent (p-dimethylaminobenzaldehyde) was added into the media to detect if indole has been made by the media. The development of pink or red layer on top of media is a positive result. Failure to see a red layer is a negative result.

3.3.5 Oxidase Test

The bacterial colonies were picked with a sterile toothpick and streaked on filter paper saturated with 0.5 % oxidase reagent (tetramethyl-p-phenylenediamine hydrochloride). Rapid appearance of dark purple color within 15 seconds was considered as a positive reaction. No change of color was considered a negative result.

3.3.6 Salt Tolerance Test

Nutrient broth was prepared individually with the presence of 0% and 8% (wt/vol) NaCl. A single bacterial colony was inoculated into the nutrient broth and incubated at 37ºC overnight. Positive results were determined by examining the turbidity.

3.3.7 Gram-staining

A single bacterial colony was inoculated with sterile loop from the nutrient agar plate. The colony was placed on the microscope slide and was spread in a circular motion. Then, heat-fix smears were performed by placing the bottom of the slide to heat approximately 30 seconds. The surface of the slide was flooded by crystal violet stain for 60 seconds. The slide was rinsed with distilled water. After that, the slide was flooded with Gram's iodine for 60 seconds and rinsed with distilled water. Then, the slide was rinsed with 95 % alcohol for 30 seconds and rinsed once again with distilled water. The slide was flooded with counterstain, Safranin for 60 seconds. The slide was washed off with distilled water and dried. Finally, the slide was observed under the oil immersion lens.

3.4 DNA extraction by boiled extraction method

1 ml of cell culture from overnight in LB was spun at 10 000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was suspended with 1 ml sterile distilled water. After centrifugation at 10 000 rpm for 5 minutes, the pellet was resuspended with 1 ml of sterile distilled water and was boiled for 10 minutes. The tubes were immediately placed on ice for 10 minutes; then the cell lysate was centrifuged and the clear supernatant was transferred into a new tube to be used as DNA template in the PCR assay.

3.5 Specific PCR amplification

PCR amplification was performed for the detection of *omp*W genes using *omp*W primer according to procedure described by Nandi *et al*. (2000).

Table 3.1 Oligonucleotide primers used to target *omp*W gene.

Target gene	Oligonucleotide sequence	Amplicon size (bp)	Reference
*omp*W	F: 5'- CAC CAA GAA GGT GAC TTT ATT GTG-3' R: 5'- GAA CTT ATA ACC ACC CGC G-3'	588	Nandi *et al*., 2000

Table 3.2 PCR reaction mixture of 25 µl volume reaction.

Reagent	Quantity per reaction (µl)
DNA template	5.0
Primer F (5pmol/µ)	1.0
Primer R(5pmol/µ)	1.0
10 mM deoxynucleotide triphosphates (dNTPs)	0.5
Taq polymerase (5U/µl)	0.2
10X reaction buffer	2.5
25 mM $MgCl_2$	2.0
Sterile distilled water	12.8
Total volume	25.0

Table 3.3 Amplification condition of PCR analysis.

Step cycle	Temperature/Time	
Initial denaturation	94ºC (3 min)	
Denaturation	94ºC (1 min)	30 cycles
Annealing	64ºC (1 min)	
Elongation	72ºC (2 min)	
Final elongation	72ºC (5 min)	

3.6 Agarose gel electrophoresis

The PCR products were resolved by electrophoresis in 1.2% agarose gel in 1X Tris-Borate-EDTA (TBE). 5µl PCR products were loaded into sample wells and voltage at 90-100 volt was used for 30-40 minutes. Then, the gel was stained with ethidium bromide (0.5µg/ml) solution for 20 minutes. The resolved bands were visualized under UV transilluminator and photographed.

CHAPTER 4

RESULTS

4.1 *Vibrio cholerae* identification

4.1.1 Bacterial Morphology on Selective Media

All the *Vibrio cholerae* strains were isolated using a selective media, namely Thiosulfate Citrate-Bile-Sucrose (TCBS) agar. *Vibrio cholerae* appears as yellowish colonies on the selective agar.

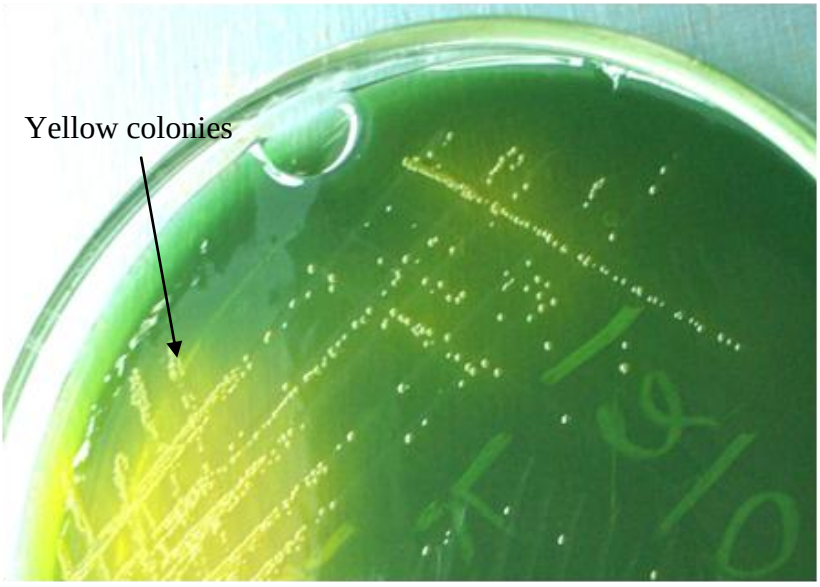

Figure 4.1 The yellow colonies of *V. cholerae* isolates on TCBS agar plate. Colonies are smooth and 2-3 mm in diameter.